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Abstract

The primary objective has been development of a rapid method for purifying extrachromosomally amplified genes (DMs) from tumor cell lines without relying on cytogenetics. DMs were purified by isolating micronuclei induced by treatment with hydroxyurea. Micronuclei were dissociated from nuclei by lysis under conditions that disaggregated intermediate filaments. Rate zonal and density centrifugation steps completely separated micronuclei from nuclei, enabling preparation of probes for fluorescence in situ hybridization to metaphase chromosomes of normal peripheral blood lymphocytes to identify the genomic regions from which the DMs were generated. Four cell lines derived from different types of tumors with DM contents spanning the range typically reported in human biopsy material (e.g., 2-50 per nucleus) were analyzed. The procedures was effectively used for all cell lines, and showed that the DMs in each were derived from the c-myc locus at 8q.24. Exclusion of chromosomes from micronuclei was shown to be due to the presence of an element of chromosome function, such as a centromere, rather than to small size. These results are significant because they provide the first rapid, sensitive method for DM purification that should be applicable to human biopsy samples.

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Introduction

Loss of genetic stability during cancer progression is a recurrent theme in oncogenesis. More than 50% of primary biopsy specimens and cell lines contain chromosomal aberrations that probably arose from defective recognition or repair of local DNA lesions, or from aberrant control of cell cycle transitions. As one example, cell cycle control mechanisms prevent gene amplification from occurring at measurable frequencies in normal cells, while it has been reported in a substantial fraction of cancer *in vivo* [1 - 5]. The overall goal of the experiments in this proposal was to develop a rapid method for identifying breast cancers containing amplified genes, and to develop effective means for eliminating amplified genes to attempt to reverse or moderate tumor phenotype.

Gene amplification can often, but not always, be detected cytogenetically. It is manifested microscopically as paired extrachromosomal acentric chromatin bodies called double minute chromosomes (DMs)[3,6], dicentric chromosomes[7,8,9], or variously sized chromosomal expansions exhibiting abnormal banding patterns such as homogeneously staining regions (HSRs)[6,10,11]. It is reasonable to assume that overexpressed amplified sequences contribute a selective or survival advantage since oncogene amplification correlates with a poor prognosis for patients with ovarian cancer (*HER-2 / neu*), breast cancer (c-*myc*, *HER-2 / neu*), neuroblastoma (N-*myc*), or small cell lung carcinoma (c-*myc*)[12]. In addition, elimination of extrachromosomally amplified drug resistance genes and oncogenes from rodent and human tumor cell lines restored drug sensitivity and decreased tumorigenicity, respectively[13 - 17]. If DMs in tumors encode genes that are rate limiting for cell growth *in vivo*, as they appear to be *in vitro*[13-15,18], then their elimination would provide a chemotherapeutic strategy targeted at a specific molecular defect uniquely found in cancer cells[19,20]. This is an attractive possibility as DMs occur frequently in clinically important neoplasms including those of the breast[21], lung[22], ovary[23], and colon[24].

Only a fraction of DMs or HSRs have been shown to contain known oncogenes. This is due to both the difficulty in obtaining adequate cytogenetic preparations and a lack of appropriate probes. The need for obtaining additional probes is demonstrated by recent comparative genomic hybridization (CGH)[4,5,25] and microdissection[26,27] analyses showing that a substantial fraction of breast cancers contain amplified sequences not detected by available molecular clones. While CGH and microdissection are yielding important insights into gene amplification and identifying new candidate oncogene loci, there is a growing need for a rapid, readily available strategy for isolating extrachromosomally amplified sequence.

We reported previously that DMs appear to be selectively entrapped in micronuclei during treatment with low concentrations of hydroxyurea (HU)[13]. This observation led us to attempt to develop a rapid and general method for micronucleus purification. The data in this Progress report show that we can use a combination of density and rate-zonal centrifugation steps to purify micronuclei that contain DNA highly enriched in DM sequences. This allowed us to generate probes for fluorescence *in situ* hybridization (FISH) to identify the normal chromosomal regions from which the DMs were generated. Furthermore, we show that selective inclusion of DMs in micronuclei is not related to their small size. Rather chromosomes are excluded from micronuclei because of the presence of a centromere or some other feature contributing to normal chromosome function. A manuscript containing this data has been written and submitted for publication, and is enclosed. To save space, the description of the data and methods are abbreviated in the following narrative, and all Figures are those in the attached manuscript.

Experimental Methods and Results:

The Statement of Work listed four objectives to be explored concurrently in the first year. First was the analysis of metaphase spreads of breast cancer cell lines and biopsy specimens to detect DMs (from month 1).. Second was elimination of DMs from cell lines to determine effects on phenotype (months 6 -onwards). Third was the identification of new candidate regions amplified in DMs in breast cancer cell lines and biopsy specimens. The Fourth Task involving Clinical Trials was eliminated due to recommendations of the review panel.

The Review Panel provided useful guidance in urging us to abandon clinical trials and focus efforts on identification and elimination of DMs, and to analyze the consequences of DM elimination. The amount of money and personnel funded to pursue such studies caused us to severely modify our goals and methods of analysis. Financial limitations imposed by the budget have led to this Project being pursued by Drs. Noriaki Shimizu and Teru Kanda (the latter has a Ph.D. in molecular biology and an M.D. with a specialty in surgical oncology related to the breast). Part time assistance has been provided by a senior research technician, Ms. Kris Clarkin.

We first attempted to employ microdissection to isolate DM sequences as proposed in the Grant, but we quickly encountered problems relating to reproducible preparation of metaphase spreads and acquiring the specialized equipment required for this technique. We traveled to Texas to learn the technique from a long-time collaborator, Dr. John McGill. We realized that we needed a technique that would be far more rapid, readily applied by those who do not have the expertise demanded by this technique, and did not rely on preparation of high quality metaphase spreads. We therefore developed a new method based on purification of micronuclei as we had previously shown micronuclei to selectively entrap DMs.

Results

Purification of hydroxyurea induced micronuclei in COLO 320DM cells

Hydroxyurea (HU) inhibits ribonucleotide reductase, thereby halting cell cycle progression in S phase when used at high concentration. Lower concentrations permit DNA replication but induce micronuclei[13]. HU effectively induces COLO 320DM cells to produce micronuclei that entrap the extrachromosomally amplified DNA at high efficiency (Fig. 1*a*). Therefore, we used COLO 320DM cells[28,29] to develop a DM purification strategy based on micronucleation.

The micronucleus purification method is described in Fig. 2*a*. We first determined the HU concentration producing maximal yield of micronuclei with minimal induction of apoptosis. This involved 3 day treatment of COLO 320DM cells with 100μM HU. Efficient dissociation of micronuclei from nuclei was accomplished by treating cells with cytochalasin B to destroy actin filaments before and during homogenization, followed by increasing the pH of the lysis buffer to 8.5 to destroy intermediate filaments[32]. We purified micronuclei in the homogenate through the following 3 steps; 1) coarse separation by velocity sedimentation to remove most nuclei, 2) centrifugation through a 1.8 M sucrose layer to remove cytoplasmic components, and 3) fractionation by velocity sedimentation to completely remove nuclei. A high concentration of sucrose was included in each buffer to aid in the separation of micronuclei and to prevent their aggregation. Furthermore, we avoided wash steps to minimize loss of micronuclei.

The data in Fig. 1 and below show that the DNA in the final micronucleus preparation was highly enriched for DM sequences. We did not detect a single intact nucleus among thousands of micronuclei. More than 90% of the 4,6-diamino-2-phenylindole (DAPI)-positive particles were micronuclei based on their size and shape. Some debris was observed that was devoid of DNA as it failed to stain with DAPI. FISH using a c-*myc* cosmid probe showed that more than 80% of the RNase-treated, propidium iodide (PI)-positive structures exhibited intense hybridization (Fig. 1*b* and *c*), indicating the successful purification of micronuclei having DMs and/or submicroscopic DM precursors (i.e., episomes[33]).

Estimating the purity of the micronucleated DM preparation

The purity of the COLO 320DM micronuclei preparation was measured using competitive PCR[34,35]. The amount of DM sequences (c-*myc*) relative to a single-copy chromosomal control sequence (β-*globin*) was estimated using an internal control for each. A fixed amount of test DNA (e.g., derived from genomic DNA or micronuclei) was amplified along with serially diluted internal standard DNA in the same tube using a single primer pair for each. We then determined the dilution of standard DNA yielding equal amounts of product from test DNA. DNA standards were prepared using human c-*myc* or β-*globin* primers to amplify a fragment of the desired size

from a non-homologous DNA source (see Methodology). This generated standard DNA and test DNA products differing slightly in size that could be amplified using a single pair of primers.

Fig. 2*b* shows a typical result obtained from a competitive PCR experiment. The data show that DNA from human WS1 diploid cells competed almost equally with the c-*myc* or β-*globin* standard DNAs, indicating that these two genes exist in equal copy numbers in the WS1 genome. On the other hand, DNA from COLO 320DM cells competed with the c-*myc* standard about 60-fold more efficiently than the β-*globin* standard DNA with its cognate test DNA. This result implies that c-*myc* is amplified approximately 60-fold relative to β-*globin*, which compares favorably to the reported value for c-*myc* amplification in this cell line determined by quantitative Southern blotting (30- to 60-fold)[14,28]. Purified micronuclear DNA from COLO 320DM cells was then subjected to competitive PCR amplification using either c-*myc* or β-*globin* primers. The DNA from micronuclei competed with the c-*myc* standard DNA about 8,000-fold more efficiently than with the β-*globin* standard DNA. This corresponds to a 128 -fold enrichment of c-*myc* sequences.

Generality of micronucleation procedure to obtain preparation enriched in DM DNA

We next examined the generality of the procedure by applying it to three additional tumor cell lines containing 4 to 16-fold amplification of c-*myc* genes. The average number of DMs per metaphase in all cell lines tested thus far ranged from a high of 44 ± 28 in COLO 320DM to a low of 3.3 ± 2.4 in the glioblastoma cell line D566 (ref. 38) (Table 1). This range encompasses that reported in many biopsy samples of human tumors[3]. Furthermore, the DMs in the medulloblastoma D425 (ref. 37) and in D566 were extremely small, and could barely be visualized using fluorescence microscopy of RNase treated and PI stained preparations. We treated these lines with 100 μM HU for 3 days and purified micronuclei as described for COLO 320DM cells. DNA from each cell line was analyzed by the competitive PCR method described above to assess the fold enrichment achieved by the micronucleation procedure. Table 1 shows that the micronucleus purification protocol produced c-*myc* enrichments ranging from 32 to 128-fold. It is important to emphasize that the micronucleation protocol employed was that derived for COLO 320DM, and was not optimized individually for each cell line. These data indicate that the protocol described in Fig. 2a can be applied to a broad diversity of cell lines derived from tumor types including promyelocytic leukemia, medulloblastoma, glioblastoma, and neuroendocrine.

Use of purified micronuclear DNA for FISH

An important goal of DM purification is the isolation of DNA of sufficient quality and quantity to prepare FISH probes to enable identification of the chromosomal location(s) that generated the DMs. To investigate whether the micronuclear DNA could be used for FISH, it was uniformly amplified by degenerate oligonucleotide-primed-PCR (DOP-PCR) as described by Telenius et al.[39] to produce probes. The specificity of the probes generated from COLO 320DM and the other cell lines listed in Table 1 to localize the corresponding sequences in metaphase chromosomes isolated from normal human peripheral blood lymphocytes (Fig. 4*a-d*). The probe from each cell line hybridized solely to the terminus of the long arm of a medium size sub-metacentric chromosome. This position is consistent with the known location of c-*myc* (8q24)[40, 41]. Thus, the enrichment obtained from each cell line was sufficient to produce a highly specific FISH probe capable of detecting the single copy locus from which the DMs were generated. The data further reveal that the DMs in each cell line contain sequences derived from only a single chromosomal location. We have recently extended the procedure to a breast cell line BSMZ. Micronuclei were induced at high frequency in this cell line, but they failed to hybridize to a distinct locus (T. Kanda, data not shown). Metaphase analysis revealed that BSMZ does not contain DMs. We are currently searching for additional breast cancer cell lines which contain DMs to ascertain their chromosomal progenitor locus.

Minichromosomes containing a functional centromere were not trapped in HU-induced micronuclei

The data presented above and elsewhere[13,16] show that DMs are preferentially captured by micronuclei. This raises the question of the mechanism underlying their apparently selective inclusion. We have begun to explore this issue by determining whether a minichromosome that is the approximate size of a typical DM is incorporated efficiently into micronuclei. We employed the CHO hybrid XEW8.2.3 (refs 42, 43) which contains a 1000-2000 kb minichromosome derived from the centromeric region of human chromosome 1. This minichromosome has a functional centromere as it exhibits high mitotic stability and is detected by a human centromere-specific probe (e.g., see Fig 4*a*).

XEW8.2.3 cells were treated with several doses of HU for 3 or 7 days to assess micronucleation frequency. Many micronuclei were induced by HU in a dose dependent manner (Fig 4*b* and *c*). Hybridization of cell preparations showed that more than 95% of nuclei exhibited a single intense hybridization signal, attesting to the high efficiency of detection afforded by this probe (Fig 4*b*). By contrast, few if any micronuclei contained the minichromosome of human centromere origin (Fig. 4*b* and *c*). These data indicate that size is not an important determinant of whether a genetic element can be incorporated into micronuclei. Rather, the results indicate that centromeric and/or other sequences involved in chromosome segregation or subnuclear localization may exclude small DNA fragments from micronuclei..

Conclusions

The work completed in the past year provides us with a rapid, efficient method for assessing whether a tumor cell line contains extrachromosomally amplified genes, and it enables localization of the chromosomal region of origin. This will enable identification of cell lines with amplification of previously known regions, and elucidation of cell lines containing potentially new oncogenes. This work provides a valuable new strategy for molecular dissection of human tumors which we intend to apply to prognosis and treatment. It also raises several new questions that we will address concerning mechanisms of genetic instability and tumor progression.

The Study Section reviewing this proposal stated that HU may not be the drug of choice due to its rapid elimination and renal toxicity at high drug concentrations. We will investigate whether agents other than HU induce micronucleation, and whether conditions can be obtained to make them more efficient than HU. Another problem that will need to be investigated prior to extending the method described herein to human biopsy material is whether normal cells that contaminate the biopsy material will contribute to background. We have started to investigate these issues and have intriguing preliminary data indicating that agents that slow S-phase induce micronuclei in tumor, but not normal cells. We will expand this research in the first part of the next grant year. This represents a change in direction from the proposed Statement of Work, but it is being done based on recommendations implicit in the summary sheet.

The work completed thus far indicates the usefulness of the DM isolation method for cell lines representing diverse tumor types. An important goal for the next year will be to establish tumors in vivo from these cell lines and develop the conditions needed to detect DMs from tumor samples obtained by biopsying these tumors. We will also attempt to scale down the procedure to make it compatible for use with fine needle biopsies often performed on breast cancer patients. Dr. Kanda's surgical training and familiarity with the micronucleus preparation procedure will make him optimally suited for such demanding experiments. Once the method has been reduced to practice on tumor samples taken from animals, we will attempt to collaborate with physicians at UCSD and/or elsewhere to obtain human biopsy material for analysis.

We have noted that all tumor cell lines we have analyzed generate micronuclei. This raises the question of the biological role of such structures. It is possible that they are normally used to purge the cell of extrachromosally amplified material, or of viruses. On the other hand it is also possible that they could serve as vehicles for transfer of genetic information between tumor cells. This would provide an effective means of accelerating tumor evolution, similar to plasmid transfer between bacteria. We will investigate this possibility in the next year using specially constructed

model episomes driven by viral replication origins. These episomes will be built to express genes that can readily be detected upon introduction into suitable recipient cells. If this is successful, we will attempt to transfer DMs between cell lines. This will be aided by our substantial collection of cell lines with DMs encoding genes that can be readily selected using drug or phenotypic selections. This represents a change in focus from the statement of work, but the exciting implications of such studies readily justify the effort at this time.

A final goal for the upcoming year, if time and resources permit, will be to assess changes in tumor phenotype upon removal of DMs. We have published that HL60 promyelocytic leukemias differentiate upon reducing c-myc gene content, and COLO320DM become less tumorigneic in nude mice. We will investigate the phenotypic consequences of removing c-myc genes from COLO320DM, and we will obtain other cell lines with amplified genes other than c-myc. We will use micronucleation to reduce gene content, isolate the DMs to define their chromosmal regions of origin, and then assess whether the treated cells differentiate or apoptose. Our primary focus will be to do this in breast cancer cell lines. If they are unavailable, we will perform trials in cell lines established from other tumor types to begin to build a data base enabling predictions of likely consequences to be made for breast cancer cells.

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Selective capture of acentric fragments by micronuclei provides a rapid method for purifying extrachromosomally amplified DNA

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[Abstract]

We present a strategy to purify amplified DNA on double minute chromosomes (DMs) to enable analysis of their prevalence and contribution to tumorigenesis. Using cells from diverse tumor types, we developed a general and rapid method to purify micronuclei that are known to entrap extrachromosomal elements. The isolated DNA is highly enriched in DM sequences and can be used to prepare probes to localize the progenitor single copy chromosomal regions. The capture of DMs by micronuclei appeared dependent on their lack of a centromere rather than their small size.

Loss of genetic stability during cancer progression is a recurrent theme in oncogenesis. More than 50% of primary biopsy specimens and cell lines contain chromosomal aberrations that probably arose from defective recognition or repair of local DNA lesions, or from aberrant control of cell cycle transitions. As one example, cell cycle control mechanisms prevent gene amplification from occurring at measurable frequencies in normal cells, while it has been reported in a substantial fraction of cancer *in vivo* [1 - 5].

Gene amplification can often, but not always, be detected cytogenetically. It is manifested microscopically as paired extrachromosomal acentric chromatin bodies called double minute chromosomes (DMs)[3,6], dicentric chromosomes[7,8,9], or variously sized chromosomal expansions exhibiting abnormal banding patterns such as homogeneously staining regions (HSRs)[6,10,11]. It is reasonable to assume that overexpressed amplified sequences contribute a selective or survival advantage since oncogene amplification correlates with a poor prognosis for patients with ovarian cancer (*HER-2 / neu*), breast cancer (c-*myc*, *HER-2 / neu*), neuroblastoma (N-*myc*), or small cell lung carcinoma (c-*myc*)[12]. In addition, elimination of extrachromosomally amplified drug resistance genes and oncogenes from rodent and human tumor cell lines restored drug sensitivity and decreased tumorigenicity, respectively[13 - 17]. If DMs in tumors encode genes that are rate limiting for cell growth *in vivo*, as they appear to be *in vitro*[13-15,18], then their elimination would provide a chemotherapeutic strategy targeted at a specific molecular defect uniquely found in cancer cells[19,20]. This is an attractive possibility as DMs occur frequently in clinically important neoplasms including those of the breast[21], lung[22], ovary[23], and colon[24].

Only a fraction of DMs or HSRs have been shown to contain known oncogenes. This is due to both the difficulty in obtaining adequate cytogenetic preparations and a lack of appropriate probes. The need for obtaining additional probes is demonstrated by recent comparative genomic hybridization (CGH)[4,5,25] and microdissection[26,27] analyses showing that a substantial fraction of breast cancers contain amplified sequences not detected by available molecular clones. While CGH and microdissection are yielding important insights into gene amplification and identifying

new candidate oncogene loci, there is a growing need for a rapid, readily available strategy for isolating extrachromosomally amplified sequence.

We reported previously that DMs appear to be selectively entrapped in micronuclei during treatment with low concentrations of hydroxyurea (HU)[13]. This observation led us to develop the rapid and general method for micronucleus purification described herein. We show that purified micronuclei contain DNA highly enriched in DM sequences. This allows preparation of probes for fluorescence *in situ* hybridization (FISH) to identify the normal chromosomal regions from which the DMs were generated. Furthermore, we show that selective inclusion of DMs in micronuclei is not related to their small size. Rather chromosomes are excluded from micronuclei because of the presence of a centromere or some other feature contributing to normal chromosome function.

Purification of hydroxyurea induced micronuclei in COLO 320DM cells

HU inhibits ribonucleotide reductase, thereby halting cell cycle progression in S phase when used at high concentration. Lower concentrations permit DNA replication but induce micronuclei[13]. HU effectively induces COLO 320DM cells to produce micronuclei that entrap the extrachromosomally amplified DNA at high efficiency (Fig. 1*a*). Therefore, we used COLO 320DM cells[28,29] to develop a DM purification strategy based on micronucleation.

The micronucleus purification method is described in Fig. 2*a*. It reproducibly gives micronuclei in higher yield and of greater purity than we could achieve using previously described methods designed to isolate microcells for use in monochromosome transfer experiments[30]. We first determined the HU concentration producing maximal yield of micronuclei with minimal induction of apoptosis. This involved 3 day treatment of COLO 320DM cells with 100μM HU. We then attempted to dissociate micronuclei from nuclei using citric acid treatment followed by separation using flow cytometry as described previously[31]. However, this protocol caused severe aggregation of micronuclei during subsequent purification steps. Efficient dissociation of micronuclei from nuclei was accomplished by treating cells with cytochalasin B to destroy actin

filaments before and during homogenization, followed by increasing the pH of the lysis buffer to 8.5 to destroy intermediate filaments[32]. We purified micronuclei in the homogenate through the following 3 steps; 1) coarse separation by velocity sedimentation to remove most nuclei, 2) centrifugation through a 1.8 M sucrose layer to remove cytoplasmic components, and 3) fractionation by velocity sedimentation to completely remove nuclei. A high concentration of sucrose was included in each buffer to aid in the separation of micronuclei and to prevent their aggregation. Furthermore, we avoided wash steps to minimize loss of micronuclei.

The data in Fig. 1 and below show that the DNA in the final micronucleus preparation was highly enriched for DM sequences. We did not detect a single intact nucleus among thousands of micronuclei. More than 90% of the 4,6-diamino-2-phenylindole (DAPI)-positive particles were micronuclei based on their size and shape. Some debris was observed that was devoid of DNA as it failed to stain with DAPI. FISH using a c-*myc* cosmid probe showed that more than 80% of the RNase-treated, propidium iodide (PI)-positive structures exhibited intense hybridization (Fig. 1*b* and *c*), indicating the successful purification of micronuclei having DMs and/or submicroscopic DM precursors (i.e., episomes[33]).

Estimating the purity of the micronucleated DM preparation

The purity of the COLO 320DM micronuclei preparation was measured using competitive PCR[34,35]. The amount of DM sequences (c-*myc*) relative to a single-copy chromosomal control sequence (β-*globin*) was estimated using an internal control for each. A fixed amount of test DNA (e.g., derived from genomic DNA or micronuclei) was amplified along with serially diluted internal standard DNA in the same tube using a single primer pair for each. We then determined the dilution of standard DNA yielding equal amounts of product from test DNA. DNA standards were prepared using human c-*myc* or β-*globin* primers to amplify a fragment of the desired size from a non-homologous DNA source (see Methodology). This generated standard DNA and test DNA products differing slightly in size that could be amplified using a single pair of primers.

Fig. 2*b* shows a typical result obtained from a competitive PCR experiment. The data show that DNA from human WS1 diploid cells competed almost equally with the c-*myc* or β-*globin* standard DNAs, indicating that these two genes exist in equal copy numbers in the WS1 genome. On the other hand, DNA from COLO 320DM cells competed with the c-*myc* standard about 60-fold more efficiently than the β-*globin* standard DNA with its cognate test DNA. This result implies that c-*myc* is amplified approximately 60-fold relative to β-*globin*, which compares favorably to the reported value for c-*myc* amplification in this cell line determined by quantitative Southern blotting (30- to 60-fold)[14,28]. Purified micronuclear DNA from COLO 320DM cells was then subjected to competitive PCR amplification using either c-*myc* or β-*globin* primers. The DNA from micronuclei competed with the c-*myc* standard DNA about 8,000-fold more efficiently than with the β-*globin* standard DNA. This corresponds to a 128 -fold enrichment of c-*myc* sequences.

Generality of micronucleation procedure to obtain preparation enriched in DM DNA

We next examined the generality of the procedure by applying it to three additional tumor cell lines containing 4 to 16-fold amplification of c-*myc* genes. The average number of DMs per metaphase in all cell lines tested thus far ranged from a high of 44 ± 28 in COLO 320DM to a low of 3.3 ± 2.4 in the glioblastoma cell line D566 (ref. 38) (Table 1). This range encompasses that reported in many biopsy samples of human tumors[3]. Furthermore, the DMs in the medulloblastoma D425 (ref. 37) and in D566 were extremely small, and could barely be visualized using fluorescence microscopy of RNase treated and PI stained preparations. We treated these lines with 100 μM HU for 3 days and purified micronuclei as described for COLO 320DM cells. DNA from each cell line was analyzed by the competitive PCR method described above to assess the fold enrichment achieved by the micronucleation procedure. Table 1 shows that the micronucleus purification protocol produced c-*myc* enrichments ranging from 32 to 128-fold. It is important to emphasize that the micronucleation protocol employed was that derived for COLO 320DM, and was not

optimized individually for each cell line. These data indicate that the protocol described in Fig. 2a can be applied to a broad diversity of cell lines derived from tumor types including promyelocytic leukemia, medulloblastoma, glioblastoma, and neuroendocrine.

Use of purified micronuclear DNA for FISH

An important goal of DM purification is the isolation of DNA of sufficient quality and quantity to prepare FISH probes to enable identification of the chromosomal location(s) that generated the DMs. To investigate whether the micronuclear DNA could be used for FISH, it was uniformly amplified by degenerate oligonucleotide-primed-PCR (DOP-PCR) as described by Telenius et al.[39] to produce probes. The specificity of the probe generated from COLO 320DM micronuclei was analyzed in two ways. It was first hybridized *in situ* to metaphase spreads of COLO 320DM cells. Fig. 1*d* shows significant hybridization to DMs, to chromosomal sites near the centromeric region of a medium sized chromosome, and to opposite arms of a single metacentric chromosome suggestive of a large chromosomal inversion. These three regions were identical to those stained by the c-*myc* cosmid DNA probe (Fig. 1*e*). Other chromosomal regions were essentially devoid of signal even if the signal was amplified using avidin-biotin sandwiches (data not shown). The probe generated from purified micronuclei DNA also brightly stained micronuclei formed in interphase COLO 320DM cells (data not shown).

Next, we used the PCR amplified probes generated from micronuclei derived from COLO 320DM and the other cell lines listed in Table 1 to localize the corresponding sequences in metaphase chromosomes isolated from normal human peripheral blood lymphocytes (Fig. 4*a-d*). The probe from each cell line hybridized solely to the terminus of the long arm of a medium size sub-metacentric chromosome. This position is consistent with the known location of c-*myc* (8q24)[40, 41]. Thus, the enrichment obtained from each cell line was sufficient to produce a highly specific FISH probe capable of detecting the single copy locus from which the DMs were generated. The data further reveal that the DMs in each cell line contain sequences derived from only a single chromosomal location.

Minichromosomes containing a functional centromere were not trapped in HU-induced micronuclei

The data presented above and elsewhere[13,16] show that DMs are preferentially captured by micronuclei. This raises the question of the mechanism underlying their apparently selective inclusion. We have begun to explore this issue by determining whether a minichromosome that is the approximate size of a typical DM is incorporated efficiently into micronuclei. We employed the CHO hybrid XEW8.2.3 (refs 42, 43) which contains a 1000-2000 kb minichromosome derived from the centromeric region of human chromosome 1. This minichromosome has a functional centromere as it exhibits high mitotic stability and is detected by a human centromere-specific probe (e.g., see Fig 4*a*).

XEW8.2.3 cells were treated with several doses of HU for 3 or 7 days to assess micronucleation frequency. Many micronuclei were induced by HU in a dose dependent manner (Fig 4*b* and *c*). Hybridization of cell preparations showed that more than 95% of nuclei exhibited a single intense hybridization signal, attesting to the high efficiency of detection afforded by this probe (Fig 4*b*). By contrast, few if any micronuclei contained the minichromosome of human centromere origin (Fig. 4*b* and *c*). These data indicate that size is not an important determinant of whether a genetic element can be incorporated into micronuclei. Rather, the results indicate that centromeric and/or other sequences involved in chromosome segregation or subnuclear localization may exclude small DNA fragments from micronuclei.

Discussion

Several useful methods have been developed in recent years to elucidate amplified sequences in tumor biopsy samples[25]. CGH can reveal the chromosomal locations from which amplified sequences in tumor cells arose[4,5], but it does not differentiate sequences amplified in DMs from those amplified within chromosomes. This is important for determining which tumors may benefit from therapeutic strategies that eliminate extrachromosomally amplified sequences. Furthermore,

this method is compromised by the presence of normal cells in the biopsy specimen. Another method requires mechanical isolation of DMs from metaphase preparations of tumor tissue[26,27]. This method requires specialized equipment and technical expertise, as well as good quality metaphase spreads which are typically difficult to obtain. Microdissection is also restricted to microscopically visible DMs, and cannot be used to obtain submicroscopic episomes known to be present in many cell lines[33]. The isolation of only a few representative DMs among the many present in a tumor cell population may also produce an underestimate of the heterogeneity in the DM population. By contrast, the method reported here avoids the problems inherent in other strategies by using micronuclei. This strategy bypasses the need for cytogenetics to obtain DNA highly enriched for DMs or episomes that can then be used as a FISH probe. As the procedure requires straightforward centrifugation protocols, it is accessible to most clinical and basic research laboratories.

We examined the effectiveness of this strategy using four different human tumor lines. The fold enrichment relative to a single copy chromosomal standard ranged from 32-128 fold. The difference in apparent enrichment may be due to the use of a single protocol for micronucleation which was not optimized for cell lines other than COLO 320DM. As the precise amount of DNA which is amplified in each cell line is not known, it is difficult to provide an accurate estimate of the absolute purity of each preparation.

The application of the DM enrichment procedure to human tumors requires several conditions to be fulfilled. First, as biopsy specimens are often contaminated with normal cells, the presence of the latter must not adversely affect the DM enrichment achieved by the micronucleation procedure. Second, it must be possible to induce diverse cell types to produce micronuclei with an acceptable frequency. Third, sufficient enrichment must be achievable to allow preparation of FISH probe from cell populations in which a minority of cells contain DMs, or where the amplification level is small. We have found that mixing COLO 320DM cells with a 100-fold excess of normal cells still enables preparation of micronuclei that generate a probe that reacts intensely with the amplified sequences (N. Shimizu, data not shown). This is expected as normal

cells typically produce micronuclei at very low efficiencies[44,45] which is not increased by HU treatment (N. Shimizu *et al.*, manuscript in preparation). The data presented here show that the micronucleation procedure optimized for COLO 320DM cells can be applied to a leukemia, medulloblastoma and a glioblastoma cell line. The enrichments obtained were sufficient to generate probes that hybridized intensely to the corresponding single copy c-*myc* locus. This indicates the general utility of the procedure for human tumors derived from different cell lineages, and for cells with a low DM incidence and amplification level. The data suggest that intense hybridization of a micronucleus generated probe to single copy sequences in metaphase chromosomes may be useful for both localizing the chromosomal progenitor locus, and for identifying cell populations with extrachromosomally amplified sequences.

The mechanisms accounting for the selective entrapment of DMs by micronuclei remain to be elucidated. Our data show that small size is not a determining factor. We consider two other alternatives to be most likely. First, the micronuclei induced by HU treatment resemble those induced by clastogenic agents in that they are highly enriched for acentric fragments [46 - 48]. It is possible that HU treatment generates DNA double-strand breaks at low efficiency by slowing replication fork progression, as reported for other drugs or genetic manipulation[49,50]. Micronuclei may be the result of reconstitution of the nuclear membrane around lagging chromosomal fragments at mitosis. A second model is that acentric fragments such as DMs reside in a different nuclear location than chromosomes. The presence of elements in ectopic nuclear environments may promote "budding" of these sequences from interphase nuclei. Consistent with this, we have observed nuclei in which DM sequences appear to bud out of the nuclear membrane to form micronuclei (N. Shimizu and G.M. Wahl, unpublished observation). Our observation that small chromosomes are effectively excluded from HU-induced micronuclei could provide the basis for determining whether functional centromeres, telomeres, or both are required for exclusion from HU-induced micronuclei. This, in turn, could provide the basis for a facile assay for structure-function analyses of centromere or telomere function.

Many of the genetic changes resident in each cancer cell genome reflect the various selections imposed on the cell during tumor progression. However, the contributions of specific alterations to tumor cell growth or survival *in vivo* remain to be elucidated, and it is uncertain how many changes detected in late stage tumors are of consequence. By contrast, the presence of acentric extrachromosomal molecules implies that one or more of the genes they encode confers a selective advantage or else they would be lost, as observed when drug selection is removed from cells containing DM-encoded drug-resistance genes[16]. The strategy described here can aid in both identifying genes encoded by DMs, and in revealing how known and previously unknown protooncogenes may contribute to particular stages of tumorigenesis.

Methodology

Cell lines. Human COLO 320DM neuroendocrine tumor cell lines[28, 29] were provided by D. D. Von Hoff (University of Texas, San Antonio, Texas) and grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). The locations of amplified c-*myc* genes to DMs was confirmed by FISH using c-*myc* cosmid DNA. Human HL-60 promyelocytic leukemia cell line[36] was obtained from American Type Culture Collection (CCL240) and cultured in RPMI 1640 supplemented with 10% FCS. Human D425 medulloblastoma cell line[37] and human D566 glioblastoma cell line[38] were provided by S.H. Bigner (Duke University Medical Center, North Carolina), and maintained in improved MEM Zinc Option Medium (Gibco) supplemented with 10 mM HEPES buffer and 10% FCS. The XEW8.2.3 cell line[42, 43] was isolated by I. Scheffler (University of California, San Diego, California) and maintained in DMEM supplemented with 10% FCS. Human WS1 diploid fibroblasts were obtained from American Type Culture Collection (CRL1502) and maintained in DMEM supplemented with 10% heat-inactivated FCS and 1 x MEM nonessential amino acids. All cells were grown at 37°C with 7% CO_2. HU (Sigma) was added to the culture when passaged. Cells were grown in the presence of 100 μM HU for 3 days (unless otherwise noted).

Purification of micronuclei. Cells ($1 \times 10^8 \sim 1 \times 10^9$ cells) grown in the presence of 100μM HU for 3days were harvested and washed twice with DMEM without serum. The cell pellet was resuspended in 20 ml of prewarmed (37 °C) DMEM containing cytochalasin B (10 μg ml^{-1}) and incubated for 30 min at 37 °C. After collection by centrifugation at 200 x g for 5 min, the cells were resuspended in 10 ml pre-chilled lysis buffer (10 mM Tris-HCl, 2 mM Mg-acetate, 3 mM $CaCl_2$, 0.32 M sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% (v / v) Nonidet P-40, 0.15 mM spermine, 0.75 mM spermidine and 10 μg ml^{-1} cytochalasin B, pH 8.5, 4 °C), and Dounce-homogenized (5 to 10 strokes using a loose-fitting pestle). The release of micronuclei from other cellular components was confirmed by mixing a small portion of the homogenate with an equal volume of phosphate buffered saline without divalent cations (PBS-) containing 2 μg ml^{-1} of DAPI and examining under a fluorescence microscope. The homogenate was mixed with an equal volume of 1.8 M Sucrose Buffer (10 mM Tris-HCl, 1.8 M sucrose, 5 mM Mg-acetate, 0.1 mM EDTA, 1mM dithiothreitol, 0.3% BSA, 0.15 mM spermine, 0.75 mM spermidine, pH 8.0, 4 °C), and a 10 ml portion was layered on top of the layers of Sucrose Buffers (20 and 15 ml containing 1.8 M and 1.6 M of sucrose, respectively) in a 50 ml-tissue culture tube. This was centrifuged in a JS-5.2 swinging bucket rotor (Beckman) at 2,000 rpm (gmax = 944) for 20 min at 4 °C. After centrifugation, fractions were collected from the top of the tube and examined by DAPI staining as described above. The upper 3 ml of the gradient usually did not contain micronuclei and was discarded. Micronuclei were recovered from the next 12 ml. Although most nuclei were pelletted, a significant amount of nuclear contamination was seen in this fraction. The fraction was layered on a 5 ml cushion of 1.8 M Sucrose Buffer and centrifuged at 14,000 rpm (gmax = 34,700) for 90 min at 4 °C using the SW40 rotor (Beckman). The supernatant was removed completely, and the pellet containing micronuclei and contaminating nuclei was collected and resuspended in 0.2 ml of 0.8 M Sucrose Buffer. The suspension was then layered on top of an 11 ml linear sucrose gradient (1.0 to 1.8 M in Sucrose Buffer) made in a 15-ml tissue culture tube, and centrifuged at 1,500 rpm (gmax = 530) for 15 min at 4 °C using JS-5.2 rotor. After centrifugation, fractions (1 ml each collected from the top) were examined by DAPI staining. More than 90% of the DAPI-

stained particles seen in fractions 1 to 4 were micronuclei of various sizes. Most of the nuclei pelleted under this condition, and absolutely no nuclear contamination was seen in the top four fractions. The micronuclei fractions were diluted 5-fold by adding PBS- and then centrifuged at 2,000 rpm (gmax = 944) for 15 min at 4 °C using JS-5.2 rotor. The pellet was suspended in a small amount of buffer (~ 0.1 ml). A portion of the purified micronuclei was fixed in methanol / acetic acid (3 / 1), and examined by FISH using a c-*myc* cosmid probe. The remaining samples were treated with proteinase K (60 μg ml^{-1}) and 0.05% Triton X-100 for 60 min at 50 °C followed by inactivation of the enzyme at 94 °C for 12 min, and then used for gene quantification by PCR or the generation of FISH probes.

Gene quantification by PCR. We quantified the levels of c-*myc* gene amplification and DM enrichment relative to the single copy β-*globin* gene on chromosome 11 using competitive PCR[34,35]. The primers used for the c-*myc* gene were myc-C, 5'd(CTG GGA TCT TCT CAG CCT AT)3' and myc-D, 5'd(ACT CCT CTC ACC ATG AAG GT)3'. The sequences of primers used for β-*globin* are IVS-I, 5'd(GTA TCA TGC CTC TTT GCA CC)3', and IVS-L, 5'd(AAG GGC CTA GCT TGG ACT CA). The primer set myc-C and myc-D amplifies 400 bps of the human c-*myc* gene intron 2, and the primer set IVS-I and IVS-L amplifies 214 bps of the human β-*globin* gene intron 2. Internal standards for each gene were generated from salmon DNA or *Saccharomyces pombe* DNA by PCR amplification using c-*myc* or β-*globin* primer pairs, respectively, by lowering the annealing temperature to 42 °C or 47 °C, respectively. The products were separated by agarose gel electrophoresis, and bands differing in size from the human DNA PCR product were excised. The excised bands were further purified by three successive rounds of PCR amplification at a higher annealing temperature (63 °C) and fractionation by agarose gel electrophoresis. The final PCR products, each of which gave a single band in agarose gel electrophoresis, were used as the internal standards in competitive PCR. Competitive PCR reactions contained equal amounts of test DNA and serial 2-fold dilutions of standard DNA. Each tube (10 μl) contained 1 x Taq buffer (Invitrogen; N for c-*myc* , J for β-*globin*), 0.2 mM of each

dNTP, 20 ng of each primer, 0.2 ml test DNA, serially-diluted known amounts of standard DNA, and 0.4 u Taq DNA polymerase (Boehringer-Manheim). The tubes were heated to 95 °C for 3 min followed by 35 cycles at 94 °C for 1 min, 63 °C for 1 min, and 72 °C for 2 min. The products were then separated by agarose gel electrophoresis, stained with ethidium bromide and the intensities of the products from the test and standard DNA were compared.

FISH. DNA in the proteinase K-treated micronuclei preparation was uniformly amplified by randomly primed PCR as reported by Telenius *et al.* [39]. Amplified products were labeled with biotin using the "BioPrime DNA Labeling System" (BRL Life Technologies) as per the manufacturer's protocol. The experiment in Fig. 1*d* employed uniformly amplified products that were FITC-labeled by the "Prime-It Fluor Fluorescence Labeling Kit" (Stratagene). c-*Myc* cosmid DNA was obtained from Yuxin Yin (Salk Institute, La Jolla, CA)[13] and labeled with biotin-14-dCTP by random hexamer extension. Biotinylated human centromere probe cocktail was purchased from Oncor. Metaphase spreads were treated with RNase (100 μg ml^{-1} in 2 x SSC, 37 °C, 60 min), and hybridized using standard conditions[51]. Hybridizations, except those to centromeric sequences, used 50 - 100 ng of probe in a 15 μl of hybridization mixture containing 50% formamide, 10% dextran sulfate, 2 x SSC, 6 μg salmon sperm DNA, 3 μg shared human placental DNA (only for the experiment of Fig. 3) and 3 μg human COT I DNA (BRL). COT I DNA was not used in the experiments of Fig. 4. The probe mixture was denatured at 75 °C for 5 min followed by 42 °C for 30 min. The slides with metaphase spreads were denatured in 70% formamide, 2 x SSC at 72 °C for 2 min, dehydrated in ice cold 70%, 85% and 100% ethanol for 3 min each, and air dried. Hybridization was done at 37 °C in a moist chamber overnight. Slides were then washed three times in 50% formamide, 2 x SSC at 45 °C for 3 min each, 3 x 3 min in 2 x SSC at 45 °C, and one time in 0.1 x SSC at 60 °C for 10 min. Slides were viewed at this point when FITC labeled probes were used. Alternatively, the hybridization signal of biotin labeled probe was detected with one layer of FITC-conjugated avidin (Vector), amplified with biotinylated anti-avidin (Vector) and a second layer of FITC-conjugated avidin. Slides were counterstained

with 0.5 μg ml^{-1} of PI in Vectashield (Vector) and examined with a Zeiss fluorescence microscope equipped with the appropriate epifluorescence filters. All photographs were taken at a magnification of 1000 x, except Fig. 1*b* which was 400 x as indicated in the legend.

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[Legends to Figures]

Fig. 1 Analysis of micronuclei and micronuclei-DM probes from COLO 320DM cells. *a*, Metaphase spread from COLO 320DM cells treated with 100 μM HU for 3 days hybridized to biotinylated c-*myc* cosmid probe and detected by FITC-avidin. The signal was amplified by a second layer of biotinylated anti-avidin and FITC-avidin. Micronucleus stained by the c-*myc* probe is shown by the arrow. *b*, *c*, Purified micronuclei from COLO 320DM cells were fixed and analyzed by FISH as in (*a*). (Magnification of *b* was 400 x, while all other photographs in this paper are 1000 x.) *d*, DNA in the purified micronuclei was uniformly amplified, FITC-labeled and hybridized to metaphases from COLO 320DM cells. Some DMs stained by the probe are indicated by small arrows. The three chromosomal regions stained by this probe are indicated by large arrows and an arrow head. *e*, For comparison to (*d*), a COLO 320 DM metaphase hybridized with c-*myc* cosmid probe. The signal was amplified as in (*a*). The same three chromosomal regions as in (*d*) are indicated by arrows and arrow head.

Fig. 2 Purification of micronuclei from COLO 320DM cells. *a*, Outline of the micronucleus purification procedure. *b*, Purity of DM sequences in the purified micronuclei. The amount of c-*myc* and β-*globin* sequences in total genomic DNA from WS1 or COLO 320DM, or from purified COLO 320DM micronuclei was quantified by competitive PCR. A constant amount of test DNA was amplified using c-*myc* or β-*globin* primer pairs in the presence of serially diluted standard DNAs. PCR products were fractionated by agarose gel electrophoresis and stained with ethidium bromide. The amount of standard DNA added to each reaction is noted above each lane. The numbers are bolded and underlined when the test and standard DNA had roughly equivalent band intensities.

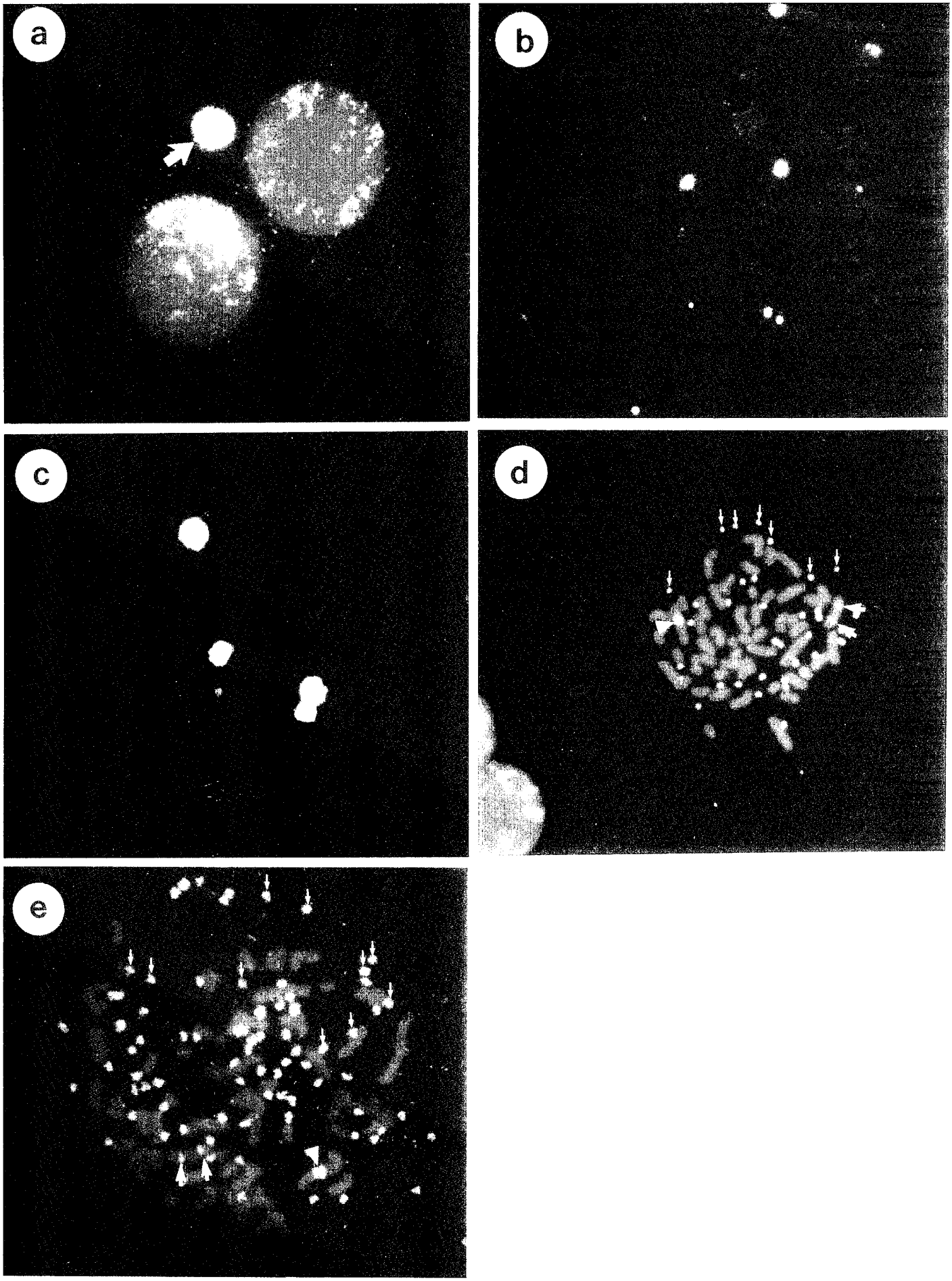
Fig. 3 Metaphases from normal human peripheral blood lymphocytes hybridized to the micronuclei probe from 4 different human tumor cell lines. Biotinylated probes were made from purified micronuclei of *a*, COLO 320DM cells; *b*, HL-60 cells; *c*, D425 cells; and *d*, D566 cells, and were hybridized to metaphases from normal human peripheral lymphocytes. The signal was amplified as in Fig. 1*a*. All probes reproducibly detected one locus (arrows) consistent with the known location of c-*myc* on 8q24.

Fig. 4 Minichromosomes containing centromere sequences were not entrapped in HU-induced micronuclei. A human centromere probe cocktail was hybridized to metaphase spreads from XEW8.2.3 cells. *a*, Minichromosome with human centromere sequence is stained by the probe in the background of Chinese hamster chromosomes as indicated by the arrow. *b*, Almost all of the hybridization signal (black arrows) seen in interphase cells was located in nuclei but not in micronuclei (white arrows). *c*, Cells were treated with various concentrations of HU for 3 or 7 days. Metaphase spreads were examined by FISH as above. The numbers of total micronuclei (black bar) and the micronuclei stained by human centromere probe (hatched bar) were scored and expressed as frequency of micronuclei (%) relative to the number of interphase nuclei scored (more than 1000 for each point).

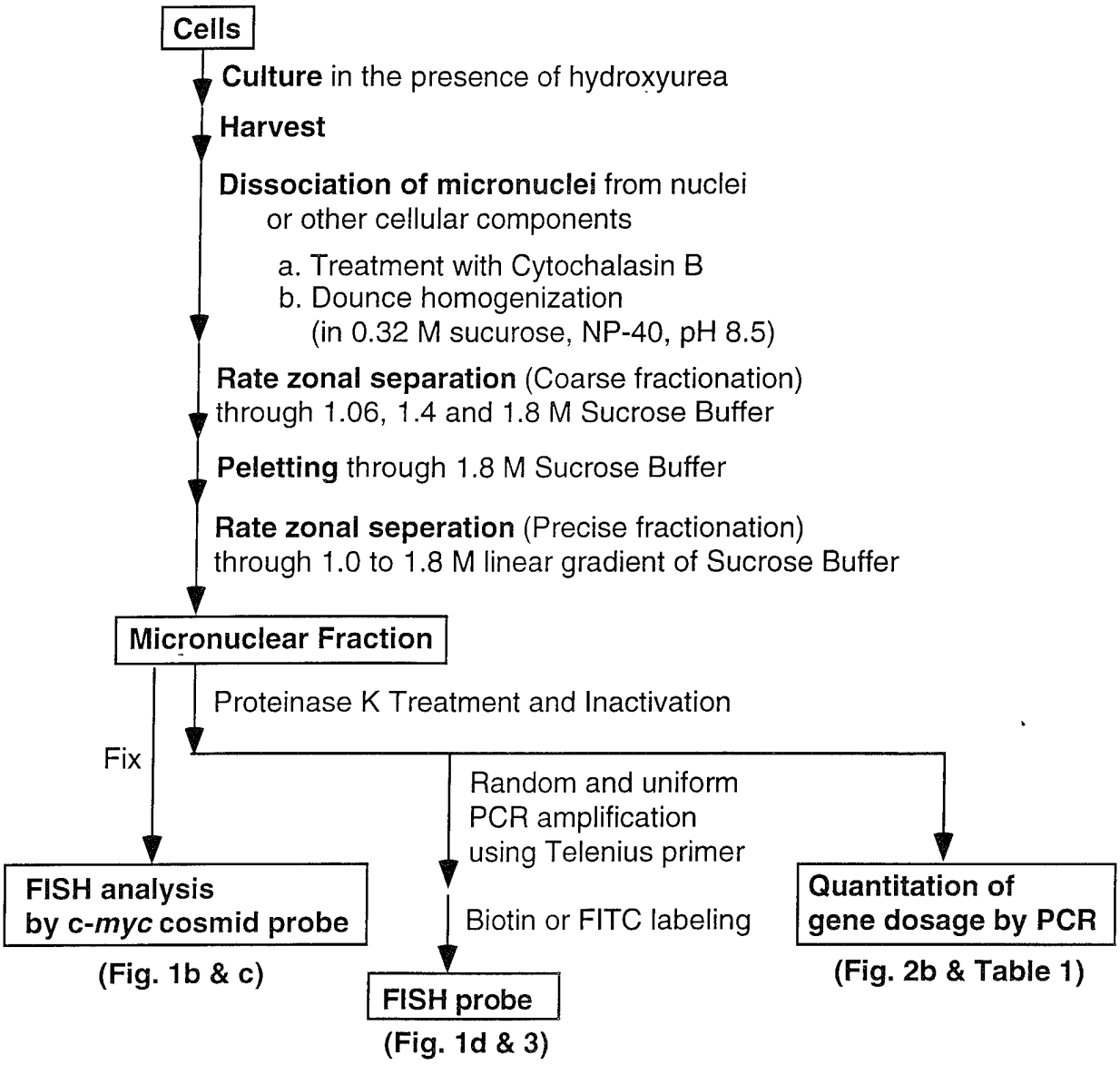
Table 1 Summary of the results showing the enrichment of DM-sequence in purified micronuclei

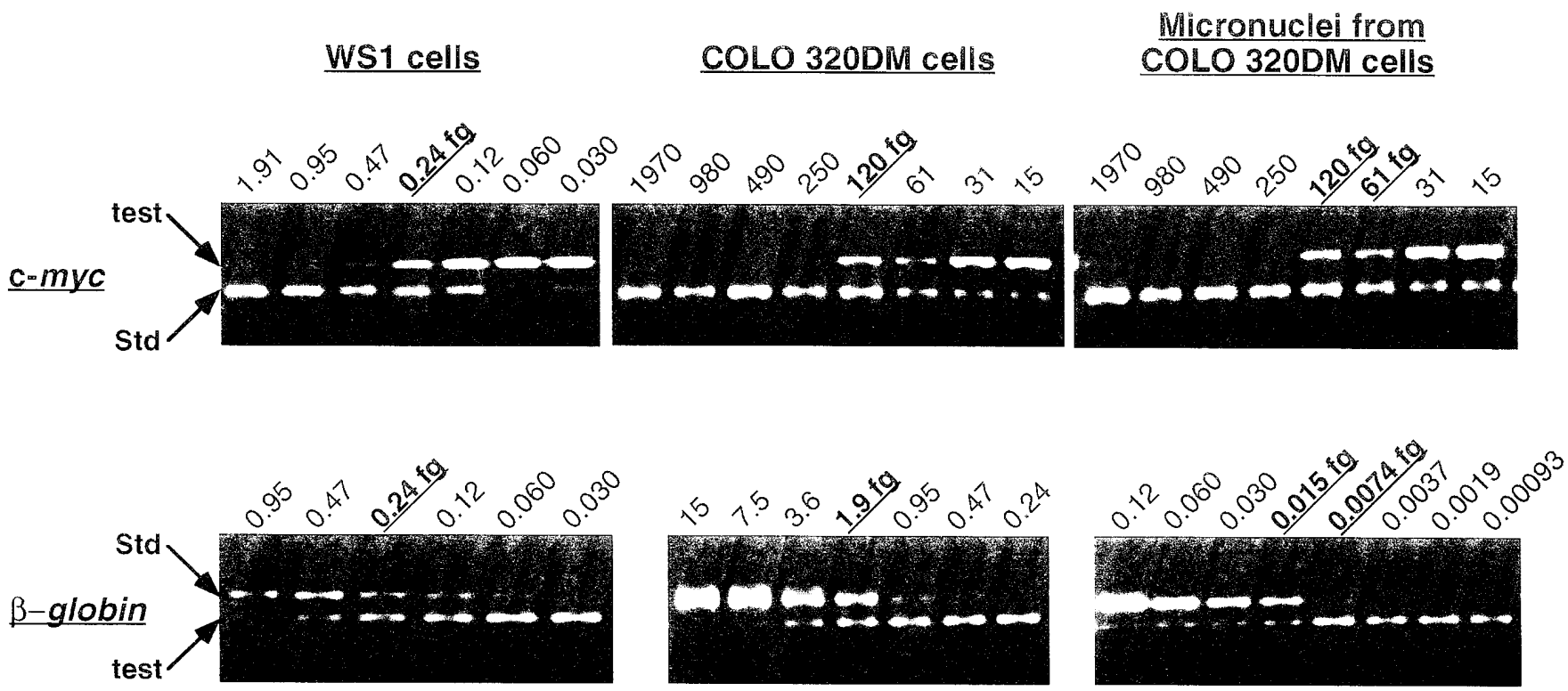
Cell Line	Tumor Type	No. of DMs per metaphase[a]	Copy No. of c-*mycc* Relative to β-*globin* [b]		Fold enrichment
			Whole Cells	Purified Micronuclei	
COLO 320DM	Colon Carcinoma (Neuroendocrine)	43.6 ± 27.6	64	8,192	128
HL-60	Promyelocytic Leukemia	4.9 ± 7.3	16	1,024	64
D425	Medulloblastoma	14.9 ± 11.4	8	256	32
D566	Glioblastoma	3.3 ± 2.4	4	256	64

[a]The number of DMs in RNase treated and PI stained metaphase was counted and expressed as mean ± standard deviation
[b]Copy numbers were determined as the same way as Fig. 2b


a
b
c
d
e

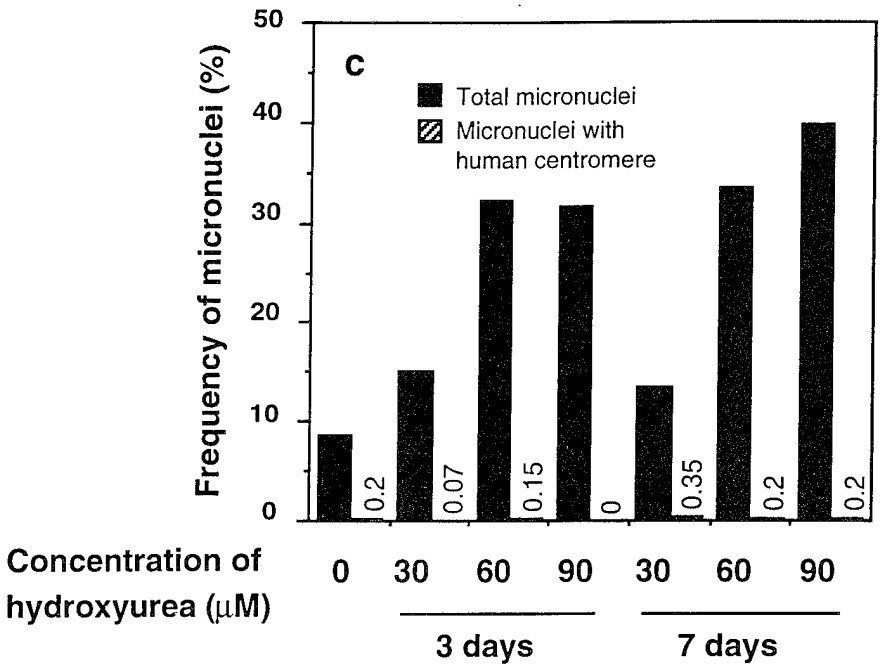
a


Cells
Culture in the presence of hydroxyurea
Harvest
Dissociation of micronuclei from nuclei or other cellular components
a. Treatment with Cytochalasin B
b. Dounce homogenization (in 0.32 M sucurose, NP-40, pH 8.5)
Rate zonal separation (Coarse fractionation) through 1.06, 1.4 and 1.8 M Sucrose Buffer
Peletting through 1.8 M Sucrose Buffer
Rate zonal seperation (Precise fractionation) through 1.0 to 1.8 M linear gradient of Sucrose Buffer
Micronuclear Fraction
Proteinase K Treatment and Inactivation
Fix
Random and uniform PCR amplification using Telenius primer
Biotin or FITC labeling
FISH analysis by c-myc cosmid probe
(Fig. 1b & c)
FISH probe
(Fig. 1d & 3)
Quantitation of gene dosage by PCR
(Fig. 2b & Table 1)


WS1 cells
COLO 320DM cells
Micronuclei from COLO 320DM cells
1.91 0.95 0.47 0.24 fg 0.12 0.060 0.030
1970 980 490 250 120 fg 61 31 15
1970 980 490 250 120 fg 61 fg 31 15
test
Std
c-myc
0.95 0.47 0.24 fg 0.12 0.060 0.030
15 7.5 3.6 1.9 fg 0.95 0.47 0.24
0.12 0.060 0.030 0.015 fg 0.0074 fg 0.0037 0.0019 0.00093
Std
test
β-globin

a
b
c
d

a
b


C
Total micronuclei
Micronuclei with human centromere
Frequency of micronuclei (%)
50
40
30
20
10
0
0.2
0.07
0.15
0
0.35
0.2
0.2
Concentration of hydroxyurea (μM)
0
30
60
90
30
60
90
3 days
7 days